IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant:	SCHLIEVERT ET AL.	Examiner:	J. HINES
Serial No.:	09/308,829	Group Art Unit:	1641
Filed:	JULY 14, 1999	Docket No.:	600.347USWO
Title:	MUTANTS OF STREPTOCOCCAL TOXIN C AND METHODS OF USE		

CERTIFICATE UNDER 37 CFR 1.8: The undersigned hereby certifies that this correspondence is being deposited in the United States Postal Service, as first class mail, with sufficient postage, in an envelope addressed to: Assistant Commissioner for Patents, Washington, D.C. 20231 on March 7, 2000.

By: Brenda House
Name: Brenda HOUSE

DECLARATION UNDER 37 C.F.R. § 1.131

Assistant Commissioner for Patents
Washington, D.C. 20231

Dear Sir:

I, Patrick M. Schlievert, declare and state the following:

1. I am one of the inventors of the subject matter of the patent application identified above, and carried on my inventive activity as an employee of the University of Minnesota.

2. I understand that the Examiner has cited the *Kline et al.* (Kline, J.B., C.M. Collins, *Infection and Immunity* 64 (3):861–869 (1996)) as prior art in prosecution of the application identified above. I understand that the *Kline et al.* reference was published in an issue of *Infection and Immunity* dated March 1996.

3. I further understand that the original filing date of my present patent application Serial No. 09/308,829 is December 6, 1996, the filing of parent provisional U.S. Patent Application Serial No. 60/033,251. The application identified above traces its pendency to this provisional application as indicated in the Declaration and Power of Attorney submitted during prosecution of this application.

4. I state that before the publication date of the *Kline et al.* reference, that is before March 1996, my co–inventors and I invented the subject matter described and claimed in the patent application identified above. As evidence, please find accompanying this declaration a photocopy of a report from my laboratory documenting at least the conception of the claimed invention before March 1996. My co–inventors and I then diligently proceeded with

implementing the present invention and filing of the priority document for the present application.

5. I further declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true, and further that the statements are made with the knowledge that willful false statements and the like are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of this application or any patent issuing thereon.

3/6/00
Date

Patrick M. Schlievert

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DECLARATION UNDER 37 C.F.R. § 1.132

Assistant Commissioner for Patents
Washington, D.C. 20231

Dear Sir:

I, Patrick M. Schlievert, declare and state the following:

1. I am an inventor of the subject matter of the patent application identified above.

2. I attended the University of Iowa, achieving a B.A. with a General Science major in 1971 and a Ph.D. in Microbiology in 1976. I was then a Post Doctoral Associate at the University of Minnesota.

3. I began my career in academia as an Assistant Professor of Microbiology and Immunology at the University of California, Los Angeles in 1979. In 1980 I moved to the University of Minnesota as an Assistant Professor of Microbiology. I became an Associate Professor in 1984 and a Full Professor in 1990. During this time, I have taught immunology courses for medical students, trained seventeen graduate students, and ten post doctoral associates, served as a University Senator, and served on committees developing the Medical School curriculum. As results of my research, I have authored more than 230 refereed scientific publications and presented more than fifty invited lectures. To support my research I have generated nearly $3,000,000 in grant support from organizations such as the National Institutes of Health and private industry. My research has focused on toxic shock syndrome and bacterial toxins.

4. I have served the Centers for Disease Control by participating in a group that defined streptococcal toxin shock syndrome. I have served the National Institutes of Health by reviewing grants for their Bacteriology and Mycology Study Section; I also review grant applications for several other granting agencies. I have testified as an expert witness on toxic shock syndrome in approximately fifty court cases. I am member of the Medical Advisory Board of the A–Fem Medical Corporation. I am also a member of the American Society for Microbiology, the Lancefield Society, the American Association of Immunologists, and the Infectious Disease Society of America.

5. I am an expert in the field of Streptococcal pyrogenic exotoxins.

6. I have read and am familiar with the Office Action dated October 4, 1999 in the patent application identified above.

7. I understand that the Examiner has suggested that the disclosure in the present patent application of structures of mutants of SPE C with mutations in one or more of 16 secondary structural features or at one or more of 39 specific amino acid residues is not sufficient to support the claims in this application to nonlethal SPE C mutants, compositions of these mutants, and methods employing these mutants. I respectfully disagree with these assertions by the Examiner for the following reasons.

8. The present patent application details experimental results for at least nine mutants of SPE C. Of the four that were tested to determine their lethality, all four were nonlethal. Further, I have conducted additional experiments confirming the disclosure of the patent application and demonstrating that these and other mutants made according to the teachings of our patent application are nonlethal.

9. In the course of these experiments confirming the teachings of our patent application, I discovered that I had made a sequencing error in the oligonucleotides employed to prepare the mutants described in Example 6 of the application as filed. Rather than changing each residue to an alanine residue, I changed each residue to a serine residue. All of the biological results for these mutants are valid and the studies were conducted properly. However, the structure of the mutant is slightly different from that reported in the application. Therefore, the results reported for tyrosine–15, tyrosine–17, and asparagine–38 should be reported as follows for Serine Mutants.

Serine Mutants

10. Three single amino acid mutants of SPE C were made: a) Y15S in which tyrosine at position 15 was changed to serine, b) Y17S in which tyrosine at position 17 was changed to serine, c) N38S in which asparagine at position 38 was changed to serine. Two double amino acid mutants of SPE C also were made: a) Y15S/N38S, b) Y17S/N38S. All mutants were constructed by use of the Quik Change method (Stratagene, La Jolla, CA) with the *speC* containing plasmid pUMN521 as template. pUMN521 contains the SPE C gene (*speC*) in pUC13 (Goshorn et al.).

11. The single amino acid mutant proteins were produced in *Escherichia coli* in 100 ml cultures. After growth in the presence of 50μg/ml ampicillin, the *E. coli* cultures were treated with 400 ml −20 °C ethanol to lyse cells and precipitate SPE C mutant proteins. pUMN521 in *E. coli* was treated comparably for use as a positive control. The precipitates were collected and restored to 1 ml. Toxin concentrations were estimated to be 25μg/ml.

12. Wild type SPE C from pUMN521 and the three single amino acid mutants were evaluated for capacity to induce rabbit splenocyte proliferation over a toxin dose range of 0.25 to $2.5x10^{-5}$ or $2.5x10^{-6}$. As indicated in Figure 7, the Y15S and N38S mutants were approximately one half as mitogenic as the wild type. The Y17S mutant was essentially nonmitogenic (Figure 8).

13. The double mutants Y15S/N38S and Y17S/N38S were also tested for ability to stimulate rabbit splenocytes compared to wild type toxin (Figure 9). Both mutants stimulated rabbit splenocytes only to one−fourth that seen by comparable amounts of wild type toxin.

14. Both double mutants were also tested for capacity to enhance endotoxin shock. Three rabbits/group were challenged intravenously with 5μg/kg of mutants or wild type toxin. After 4 hours, the same animals were challenged with 10μg/kg *Salmonella typhimurium* endotoxin (1/50 LD_{50}). Deaths were recorded over a 48 hour time period (Table 1). As indicated, neither double mutant caused lethality in the rabbits.

Table 1: SPE–C Serine Mutants are Nonlethal and Reduce Endotoxin Shock

Treatment Protein	Number Dead / Total Rabbits tested
SPE C wild type	3/3
Y15S/N38D	0/3
Y17S/N38D	0/3

Note: In the study reported in Table 1, all rabbits were challenged intravenously with 5μg/kg protein and then 4 hours later with endotoxin (10μg/kg).

15. One week after challenge of the rabbits used in Table 1, the animals were euthanized and examined for gross tissue damage. All organs, including liver, spleen, kidneys, lungs and heart appeared normal. This is consistent with the lack of toxicity of the double mutants.

16. Three rabbits/group were also immunized with two weekly doses of 25μg of SPE C double mutants emulsified in Freund's incomplete adjuvant. The animals were then rested for 5 days. 0.5 ml of blood was collected from each animal and pooled for collection of Y15S/N38S and Y17S/N38S sera. The sera from these pools was compared to preimmune pooled serum by peroxidase based ELISA (Hudson and Hay reference) for antibodies against purified streptococcal derived wild type SPE C. Table 2 summarizes the results of the ELISA.

Table 2: SPE–C Serine Mutants are Immunogenic.*

	Sample tested	ELISA titer:
Y15S/N38D	Preimmune	<10*
	Immune	80
Y17S/N38D	Preimmune	<10
	Immune	80

*Sera to be tested for antibody were diluted 2–fold beginning at 1:10. The titer of antibody is the reciprocal of the last dilution that gave an absorbency at 490 nm of 0.1 or greater.

17. The immunized animals were then challenged with 5μg/kg of wild type SPE C and then 4 hours later 10μg/kg of *Salmonella typhimurium* endotoxin as a test for capacity to immunize against lethality. The data presented in Table 3 indicates the animals were protected from challenge and were thus immune to SPE C.

Table 3: SPE–C Serine Mutants Elicit A Protective Immune Response

Rabbit Group	Number Dead/Total Tested
Nonimmune	2/2
Y15S/N38S immune	0/3
Y17S/N38S immune	0/3

Additional Experiments Confirming the Teaching of our Patent Application

18. To provide further confirmation that the teaching of the present application yields nonlethal SPE C mutants, I constructed the following alanine mutants. These are the alanine mutants that I believed I had constructed for the studies reported in the patent application. These alanine mutants proved to be nonlethal.

19. Two single amino acid mutants of SPE C were made: a) Y15A in which tyrosine at position 15 was changed to alanine and b) Y17A in which tyrosine at position 17 was changed to alanine. Two double amino acid mutants of SPE C also were made: a) Y15A/N38D and b) Y17A/N38D, in which N38D designates a mutant in which asparagine at position 38 was changed to aspartic acid. All mutants were constructed by use of the Quik Change method (Stratagene, La Jolla, CA) with the *speC* containing plasmid pUMN521 as template. pUMN521 contains the SPE C gene (*speC*) in pUC13 (Goshorn et al.).

20. The single amino acid mutant proteins were produced in *Escherichia coli* in 100 ml cultures. After growth in the presence of 50μg/ml ampicillin, the *E. coli* cultures were treated with 400 ml –20 °C ethanol to lyse cells and precipitate SPE C mutant proteins. pUMN521 in *E. coli* was treated comparably for use as a positive control. The precipitates were collected and restored to 1 ml. Toxin concentrations were estimated to be 25μg/ml.

21. Wild type SPE C from pUMN521 and the two single amino acid mutants were evaluated for capacity to induce rabbit splenocyte proliferation over a toxin dose range of 0.25 to $2.5x10^{-5}$ or $2.5x10^{-6}$ (Figure 11). The Y15A and Y17A mutants were essentially nonmitogenic.

22. The double mutants Y15A/N38A and Y17A/N38A were also tested for ability to stimulate rabbit splenocytes compared to wild type toxin (Figure 12). Both mutants stimulated rabbit splenocytes only to less than one–sixth that seen by comparable amounts of wild type toxin.

23. Each of the mutants was also tested for capacity to enhance endotoxin shock. Two rabbits/group were challenged intravenously with about 25μg/kg of mutants or 5μg/kg wild type toxin (derived from Group A Streptococcal strain T18P). Temperatures were monitored

rectally (Table 4). Then the rabbits were challenged intravenously with 5μg/rabbit of Salmonella typhimurium endotoxin. Deaths were recorded over a 48 hour time period (Table 4). As indicated, neither double mutant nor the Y15A mutant caused lethality in the rabbits. Each of the mutants reduced fever.

Table 4: SPE–C Alanine Mutants are Nonlethal and Reduce Fever and Endotoxin Shock

SPE C	Pyrogenicity - –°C at 4 hr.	Dead/Total
Wild Type	1.65	2/2
Y15A	0.3	0/2[a]
Y17A	0.65	½
Y15A/N38D	0	0/2[a]
Y17A/N38D	1.25	0/2[a]

[a] Animals did not show signs of streptococcal toxic shock syndrome, but rather, remained healthy

24. Employing the methods described in the present patent application, I constructed a triple mutant Y15A/H35A/N38D. This mutant was evaluated for its pyrogenicity, lethality, and protective ability when used as a vaccine. These results are reported in Tables 5–7. The triple mutant was not pyrogenic, was substantially nonlethal, and showed a protective effect when used as a vaccine. These results confirm that the mutants described in our patent application are nonlethal and can be made by methods described in that application.

Table 5: SPE–C Double and Triple Mutants Reduce Fever

Protein tested	Increased fever response (Δ°C±SEM)[a]			
	Proteins administered in Miniosmotic pumps[b]	P–value[c]	Proteins administered intravenously[d]	P–value[c]
Wild type	ND[e]		1.1 (± 01.18)	
Y15A/N38D	ND		0.3 (± 0.13)	0.001
Wild type	1.14 (± 0.25)		0.5 (± 0.42)	
Y15A/H35A/N38D	−0.3 (± 0.22)	0.002	−0.66 (± 0.39)	0.032

[a] Five rabbits per group were administered 5 μg/kg/ml i.v. of wild–type or mutant SPE C proteins in PBS.
[b] Temperatures were recorded on day 0 and day 2. 500 μg of each protein was administered in subcutaneously implanted miniosmotic pumps.
[c] Student's *t*–Test.
[d] Temperatures were recorded at time 0 h and 4 h. Rabbits were treated with 5 μg/ml•kg of SPE C or various mutant proteins suspended in PBS administered iv.
[e] Not determined.

Table 6. SPE–C Double and Triple Mutants are Nonlethal and Reduce Endotoxin Shock.

Protein tested[a]	No. of rabbits dead/total no. of rabbits			
	Miniosmotic pump[b] model	P–value[c]	Enhancement of endotoxin in shock model[d]	P–value
Wild type	3/3		5/5	
Y15A/N38D	0/5	0.018	0/5	0.004
Wild type	5/5		4/4	
Y15A/H35A/N38D	0/5	0.004	0/5	0.008

[a] Five rabbits per group were administered 5 μg/kg/ml i.v. of each wild–type or mutant SPE C molecules in PBS.
[b] 500 μg of each protein was administered in subcutaneously implanted miniosmotic pumps.
[c] Student's *t*–Test.
[d] Rabbits were treated with 5 μg/ml•kg of SPE C or various mutant proteins suspended in PBS administered iv. After 4 h, 10 μg/ml•kg of purified *Salmonella typhimurium* endotoxin suspended in PBS was given iv, and mortality was recorded over 48 h.

Table 7: SPE–C Double and Triple Mutants are Immunogenic and Elicit A Protective Immune Response

Immunizing agent	Preimmune[a] titer	Immune titer	Number dead	P–value[d]
none	20	20	5/5[b]	
Y15A/N38D	208	9600	0/5	0.008
none	208	320	5/5[c]	
Y15A/H35A/N38D	144	11264	0/5	0.008

[a] Rabbits were bled prior to administration of the first immunization. The average anti–SPE C titer of 5 rabbits is reported.
[b] The endotoxin enhancement model of TSS was used.
[c] The miniosmotic pump model of TSS was used.
[d] Fisher's exact test.

Deletion Subcloning

25. In his prior art rejection, the Examiner cited a publication of which I am a co–author, *Goshorn et al.* (*Infection and Immunity* 56 (9):2518–2520 (1988)). The Examiner seems to have interpreted a statement about "deletion subclones" in this paper to mean that I had made mutants of SPE C. I am uncertain how the creation of deletion subclones while cloning and sequencing the gene for a wild type SPE–C relates to the present claims. Deletion subcloning refers to a procedure in which the ends of a large piece of DNA are trimmed away to provide a smaller piece of DNA with an intact coding sequence. Each of these deletion subclones included an intact SPE–C wild type coding sequence. That is, the deletion subcloning was a tool I employed to clone and sequence the wild type SPE C. I made no mutants of SPE C. The *Goshorn et al.* reference does not describe any mutant of SPE C.

26. I further declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true, and further that the statements are made with the knowledge that willful false statements and the like are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of this application or any patent issuing thereon.

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